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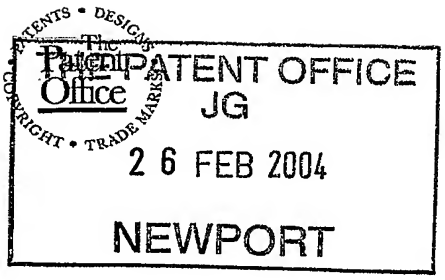

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28FEB04 E876698-1 D10100
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

HAMLET Ltd
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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

08816928001

4. Title of the invention

Therapeutic Treatment

5. Name of your agent *(if you have one)*

Carol P. Greaves et al.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Therapeutic Treatment

The present invention relates to a method of treatment of mucosal cancer such as bladder cancer, or for the treatment of melanoma, and to the use of biologically active complexes in the preparation of medicaments for the treatment of malignant mucosal tumours as well as melanomas.

HAMLET (human α-lactalbumin made lethal to tumour cells) (formerly known as MAL) is an active folding variant of alpha-lactalbumin (also represented as α-lactalbumin) that induces apoptosis in transformed cells but spares healthy differentiated cells (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, 97, 4221-6). HAMLET has been shown to bind to the surface of tumour cells, to translocate into the cytoplasm and to accumulate in cell nuclei, where it causes DNA fragmentation (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, 97, 4221-6). Biologically active complexes of this type, obtained from milk and particularly human milk, together with their use as antibacterial agents is described for example in EP-0776214.

To date, work reported with HAMLET has indicated that *in-vitro*, transformed cells are susceptible to HAMLET, which suggests that there it has an application in cancer therapy. The correlation with effects seen *in vitro* and those observed *in vivo* is not always straightforward however, in particular as conditions found *in vivo* vary depending upon the nature and position of the tumour. For instance, the different conditions found in different organs of the body can affect the stability and therefore the efficacy of any therapeutic reagent.

The applicants have found that HAMLET and complexes of this type produce unexpectedly good results when used in the treatment of mucosal tumours, particularly bladder cancer.

According to the present invention, there is provided the use of a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the treatment of mucosal cancers.

The conditions found at mucosal surfaces can be quite unique in terms of properties such as p.H. and the like. Mucosal surfaces are found inter alia in the nasal passages, in the mouth, throat, oesophagus, lung, stomach, colon, vagina and bladder. Particular mucosal surfaces that may be treated with in accordance with the invention include throat, lung, colon and bladder surfaces which tumours. The invention is particularly applicable to the treatment of bladder cancer.

As used herein, the term "HAMLET" refers to a biologically active complex of α-lactalbumin, which is either obtainable by isolation from casein fractions of milk which have been precipitated at pH 4.6, by a combination of anion exchange and gel chromatography as described for example in EP-A-0776214, or by subjecting α-lactalbumin to ion exchange chromatography in the presence of a cofactor from human milk casein, characterized as C18:1 fatty acid as described in WO 99/26979.

The α-lactalbumin may be from various mammalian sources including human, bovine, sheep and goat milk, but is preferably human or bovine, and most preferably human. Recombinant forms of the protein may also be employed.

It has also been found that other reagents and specifically lipids such as oleic acid, are useful in the conversion of human α-lactalbumin to HAMLET. In particular, it has been reported previously that oleic acid (C18:1:9cis) is required for HAMLET production (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6). More recently, it has been found that other fatty

acids may act as co-factors in a similar way. Optimal cofactors for the conversion of α-lactalbumin to HAMLET are C18:1 fatty acids with a double bond in the cis conformation at position 9 or 11.

α-Lactalbumin is a 14.2 kDa globular protein with four α-helices (residues 1-34, 86-123) and an anti-parallel β-sheet (residues 38-82), linked by four disulphide bonds (61-77; 73-91; 28-111 and 6-120) (K. R. Acharya, et al., (1991) *J Mol Biol,* **221**, 571-81). The native conformation of α-lactalbumin is defined by a high affinity Ca^{2+} binding site, co-ordinated by the side chain carboxylates of Asp82, Asp87 and Asp88, the carbonyl oxygens of Lys79 and Asp84, and two water molecules (K. R. Acharya, et al., (1991) *J Mol Biol,* **221**, 571-81). The protein adopts the so called apo-conformation found in HAMLET when exposed to low pH, or in the presence of chelators, that release the strongly bound Ca^{2+} ion (D. A. Dolgikh, et al., (1981) *FEBS Lett,* **136**, 311-5; K. Kuwajima, (1996) *Faseb J,* **10**, 102-09).

In order to form biologically active complexes, α-lactalbumin generally requires both a conformational or folding change as well as the presence of a lipid cofactor. The conformational change is suitably effected by removing calcium ions from α-lactalbumin. In a preferred embodiment, this is suitably facilitated using a variant of α-lactalbumin which does not have a functional calcium binding site.

Biologically active complexes which contain such variants are encompassed by the term "modifications" of HAMLET as used herein. However, the applicants have found that, once formed, the presence of a functional calcium binding site, and/or the presence of calcium, does not affect stability or the biological activity of the complex. Biologically active complexes have been found to retain affinity for calcium, without loss of

activity. Therefore complex of the invention may further comprise calcium ions.

Thus in particular, the invention uses a biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilises the complex in a biologically active form, provided that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

Suitably the cofactor is a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration.

In a particular convenient embodiment, the biologically active complex used in the invention comprises
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and
(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been released or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

As used herein the expression "variant" refers to polypeptides or proteins which are homologous to the basic protein, which is suitably human or bovine α-lactalbumin, but which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino

acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical, preferably at least 70%, even more preferably 80% or 85% and, especially preferred are 90%, 95% or 98% or more identity.

When comparing amino acid sequences for the purposes of determining the degree of identity, programs such as BESTFIT and GAP (both from Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention when discussing identity of sequences, the comparison is made by alignment of the sequences along their whole length.

The term "fragment thereof" refers to any portion of the given amino acid sequence which will form a complex with the similar activity to complexes including the complete α-lactalbumin amino acid sequence. Fragments may comprise more than one portion from within the full length protein, joined together. Portions will suitably comprise at least 5 and preferably at least 10 consecutive amino acids from the basic sequence.
Suitable fragments will be deletion mutants suitably comprise at least 20 amino acids, and more preferably at least 100 amino acids in length. They include small regions from the protein or combinations of these.

The region which forms the interface between the alpha and beta domains is, in human α-lactalbumin, defined by amino acids 34-38 and 82-86 in the structure. Thus suitable fragments will include these regions, and preferably the entire region from amino acid 34-86 of the native protein.

In a particularly preferred embodiment, the biologically active complex comprises a variant of α-lactalbumin in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional.

It has been found that in bovine α-lactalbumin, the calcium binding site is coordinated by the residues K79, D82, D84, D87 and D88. Thus modification of this site or its equivalent in non-bovine α-lactalbumin, for example by removing one of more of the acidic residues, can reduce the affinity of the site for calcium, or eliminate the function completely and mutants of this type are a preferred aspect of the invention.

The Ca^{2+}-binding site of bovine α-lactalbumin consists of a 3_{10} helix and an α-helix with a short turn region separating the two helices (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581). It is flanked by two disulfide bridges making this part of the molecule fairly inflexible. Five of the seven oxygen groups that co-ordinate the Ca^{2+} are contributed by the side chain carboxylates of Asp82, 87 and 88 or carbonyl oxygen's of Lys79 and Asp84. Two water molecules supply the remaining two oxygen's (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581).

Site directed mutagenesis of the aspartic acid at position 87 to alanine (D87A) has previously been shown to inactivate the strong calcium-binding site (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654) and the mutant proteins adopted the apo- conformation.

Therefore in a particular embodiment, the aspartic acid residue at amino acid position 87 within the bovine α-lactalbumin protein sequence is mutated to a non-acidic residue, and in particular a non-polar or uncharged polar side chain.

Non-polar side chains include alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred examples is alanine. Uncharged polar side chains include asparagine, glutamine, serine, threonine or tyrosine.

In order to minimize the structural distortion in the mutant protein, D87 has also been replaced by an asparagine (N) (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789), which lacks the non-compensated negative charge of a carboxylate group, but has the same side chain volume and geometry. The mutant protein (D87N) was shown to bind calcium with low affinity ($K_{-Ca}2 \times 10^5 M^{-1}$) (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). Such a mutant forms an element of the biologically active complex in a further preferred embodiment of the invention.

Thus particularly preferred variants for use in the complexes of the invention are D87A and D87N variants of α-lactalbumin, or fragments which include this mutation.

This region of the molecule differs between the bovine and the human proteins, in that one of the three basic amino acids (R70) is changed to S70 in bovine α-lactalbumin thus eliminating one co-ordinating side chain. It may be preferable therefore, that where the bovine α-lactalbumin is used in the complex of the invention, an S70R mutant is used.

The Ca^{2+} binding site is 100% conserved in α-lactalbumin from different species (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581), illustrating the importance of this function for the protein. It is co-ordinated by five different amino acids and two water molecules. The side chain carboxylate of D87 together with D88 initially dock the calcium ion into the cation-binding region, and form internal hydrogen bonds that stabilise the

structure (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654). A loss of either D87 or D88 has been shown to impair Ca2+ binding, and to render the molecule stable in the partially unfolded state (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654).

Further, mutant proteins with two different point mutations in the calcium-binding site of bovine α-lactalbumin may be used. For example, substitution of the aspartic acid at position 87 by an alanine (D87A) has been found to totally abolish calcium binding and disrupt the tertiary structure of the protein. Substitution of the aspartic acid by asparagine, the protein (D87N) still bound calcium but with lower affinity and showed a loss of tertiary structure, although not as pronounced as for the D87A mutant (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). The mutant protein showed a minimal change in packing volume as both amino acids have the same average volume of 125Å^3, and the carboxylate side chain of asparagines allow the protein to co-ordinate calcium, but less efficiently (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). Both mutant proteins were stable in the apo-conformation at physiologic temperatures but despite this conformational change they were biologically inactive. The results demonstrate that a conformational change to the apo-conformation alone is not sufficient to induce biological activity.

The structure of α-lactalbumin is known in the art, and the precise amino acid numbering of the residues referred to herein can be identified by reference to the structures shown for example in Anderson et al. supra. and Permyakov et al supra.

The medicaments produced in accordance with the invention are suitably pharmaceutical compositions in a form suitable for topical administration to the particular malignant mucosal tumour being treated. For instance, the composition may be in a form which is suitable for instillation into the bladder, where

bladder cancer is the being treated. These may include the commonly known carriers, fillers and/or expedients, which are pharmaceutically acceptable. Suitably however, the composition instilled into the bladder will comprise a solution of the active agent in sterile water or saline.

Topical solutions or creams suitably contain an emulsifying agent for the protein complex together with a diluent or cream base may be more suitable for application to other malignant mucosal tumours. Such formulations can be applied directly to the tumour.

In addition, such topical compositions may be applied to treat malignant skin tumours, in particular melanoma. The applicants have found that HAMLET is particularly effective against melanoma cells. The use of these compositions in this way forms a further aspect of the invention.

The daily dose of the active compound varies and is dependant on the patient, the nature of the cancer being treated etc. in accordance with normal clinical practice. As a general rule from 200mg to 1g/dose of the biologically active complex is used for administration per day, preferably by intra-vesical instillation, over a period of at least 3 and preferably at least 5 days. In particular a dosage regime comprising 750mg HAMLET per day for 5 days has proved beneficial.

The applicants have carried out studies on the effect of topical HAMLET treatment on bladder cancer. As reported below, the effects following intra-vesical instillation were extremely good.

In a further aspect of the invention, there is provided a method for treating mucosal cancers and in particular bladder cancer which comprises administering to a patient in need thereof, a biologically active complex of α-lactalbumin, selected from

HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these. The complex is suitably administered intra-vesically.

Preferred examples of the biologically active complex are illustrated above. Preferably the biologically active complex is administered in the form of a topical composition, also as described above.

The invention will now be particularly described by way of example with reference to the accompanying Figure which shows an endoluminal photograph of a bladder cancer, taken before and after treatment in accordance with the invention.

Intra-Vesical Instillation Of Hamlet In Patients With Cancer Of The Urinary Bladder

Preparation of substance and randomisation of patients

Donors of breastmilk were non-smokers and were screened for HIV prior to preparation of HAMLET. Alpha-lactalbumin was purified from human milk whey by ammonium sulphate precipitation followed by phenyl-Sepharose chromatography and size-exclusion chromatography. Excess milk from the hospital milk bank was used according to regulations for administration to premature babies. HAMLET was generated from native α-lactalbumin on an oleic acid conditioned ion-exchange chromatography column, as described in the literature. The eluted fractions were dialysed against distilled water, lyophilised and stored at -20°C.

Furthermore, HAMLET was screened for bacterial contamination and was stored as dry substance in -20°C.

Study design:

Patients awaiting surgery for a newly diagnosed, or recurrent uro-epithelial cancer of the urinary bladder, were invited to participate in the study. After informed consent the patients were subjected to cystoscopy to assess the tumour size and to

document the lesion with endoluminal photography. After treatment and prior to surgery, cystoscopy was repeated to re-assess tumour size and endoluminal photography was carried out.

Intra-vesical instillation of HAMLET was performed in the out-patient clinic under close surveillance. The instillations were given once daily, and repeated for five days. After urethral catheterisation the bladder was completely emptied and the urine was collected for analysis. HAMLET (25mg/ml, 30ml) was deposited in the bladder, the catheter removed, and the patients were asked to too keep the instillation for at least for two hours. To decrease the diuresis the patients were asked to avoid fluid intake for four hours before, and immediately after the instillation. Urine samples were provided prior to, and from the first voided urine after each instillation.

The HAMLET instillations were scheduled without interrupting or delaying the routine handling of the patients.

Patients: Seven male patients were included in the study. Based on standard tumour classification, the patients were assigned to three groups, A, B and C.

Patients A1 and A2 (92 and 86 years old) had poorly differentiated muscle invasive uro-epithelial cancer of the urinary bladder (T4g3). Due to their high age, these patients had been subjected to palliative measurements, such as repeated trans-urethral resections of the tumour (T-TUR), analgetics, and clinical observation. Both patients suffered mild lower urinary tract symptoms with frequency and urge, but they were otherwise healthy despite their high ages.

Patients B1, B2, B3, and B4 (37, 75, 70, 82 years old respectively) had superficial papillomatous bladder tumours (TAg1-2). Patients B1 and B3 had newly diagnosed tumours, and patients B2 and B4 had recurrencies of previously known highly

differentiated, superficial bladder tumours (TAg1). Patients B1 and B4 were healthy except for their tumour, while patient B2 suffered high blood pressure in combination with cardio-sclerosis. Patient B3 had high blood pressure and chronic bronchitis. Patient C1 (72 years) had previously known multi-focal manifestations of cancer in situ (CIS) of the urinary bladder. He had been subjected to intra-vesical instillations of Bacille Calmette Guerin (BCG) one year prior to inclusion. Bladder biopsies had initially shown response to the BCG treatment. Prior to inclusion recurrence of CIS had been diagnosed in biopsy specimens. This patient was otherwise healthy.

Treatment outcome:

All patients were given daily intra-vesical instillations of HAMLET on five consecutive days. None of the patients experienced any side effects of the instillations, and there were no reaction in systemic inflammatory parameters like CRP, fever or peripheral neutrophil counts.

Patients A1 and A2 had difficulties keeping the HAMLET solution in the bladder, due to lower urinary tract dysfunctions. The therapeutic effect could not be evaluated. Patient B1 showed a nearly complete reduction of the tumour after the five daily HAMLET instillations.

Patient B2 showed a small reduction in tumour size, but a marked change in the tumour character. Prior to treatment the tumour was brittle and bled on contact, but after treatment, the surface was "dry". Patient B3 carried a papillomatous tumour on the left bladder wall that was too big to be captured in one photograph. After the intravesical HAMLET instillations, the ocular tumour size assessment showed a reduction in size of ~ 50%. Patient B 4, had two small exophytic tumours on the left bladder neck. There was no apparent reduction in tumour size, but a marked change in tumour character with surface atrophy.

Patient C1 was difficult to evaluate due to the absence of exophytic tumour growth. Prior to the HAMLET instillations 3/3 bladder biopsies ("mapping") showed cancer in situ, but after the 5 day treatment, only 1/3 biopsies was positive.

TUNEL positive, apoptotic cancer cells were detected. Biopsies from macroscopically healthy bladder mucosa were taken from five of the patients. There was no effect of the HAMLET treatment identified in these biopsies.

We conclude that HAMLET treatment induces apoptosis in bladder cancer cells and significanly influences the volume and macroscopic appearance of the tumour.

Claims

1. The use of a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the treatment of mucosal cancers.

2. The use according to claim 1 wherein the mucosal cancer is bladder cancer.

3. The use according to claim 1 or claim 2 wherein the biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilises the complex in a biologically active form, provided that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

4. The use according to claim 3 wherein the cofactor is a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration.

5. The use according to any one of claims 1 to 4 wherein the biologically active complex comprises HAMLET, which is obtainable either by isolation from casein fractions of milk which have been precipitated at pH 4.6, by a combination of anion exchange and gel chromatography, or by subjecting α-lactalbumin to ion exchange chromatography in the presence of a cofactor from human milk casein, characterized as C18:1 fatty acid.

6. The use according to any one of claims 1 to 4 wherein the biologically active complex of α-lactalbumin comprises

(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and

(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been removed or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

7. The use according to claim 6 wherein the biologically active complex includes a variant of α-lactalbumin in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional.

8. The use according to claim 7 wherein the variant has a mutation at one of the amino acids equivalent to K79, D82, D84, D87 and D88 of bovine α-lactalbumin.

9. The use according to claim 8 wherein the modification is at D87 which includes a variant of α-lactalbumin having a D87A or D87N variants.

10. The use according to any one of claims 1 to 4 wherein the biologically active complex comprises a fragment of α-lactalbumin or a variant thereof, and where the fragment includes the entire region from amino acid 34-86 of the native protein.

11. The use according to any one of the preceding claims wherein the α-lactalbumin is human or bovine α-lactalbumin or a variant of either of these.

12. The use according to claim 11 wherein the α-lactalbumin is human α-lactalbumin.

13. The use according to claim 11 wherein the α-lactalbumin is mutant bovine α-lactalbumin which includes an S70R mutation.

14. A method for treating a mucosal tumour which comprises administering to said tumour in a patient in need thereof, a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these.

15. A method according to claim 14 wherein the mucosal tumour is bladder cancer.

16. A method according to claim 15 wherein the biologically active complex is administered by intra-vesical instillation.

17. A method according to claim 16 wherein from 200mg to 1g of the biologically active complex is administered in a single dosage unit.

18. A method according to claim 17 wherein the dosage unit is repeated on at least 5 days.

19. A method according to claim 18 wherein the dosage is given on consecutive days.

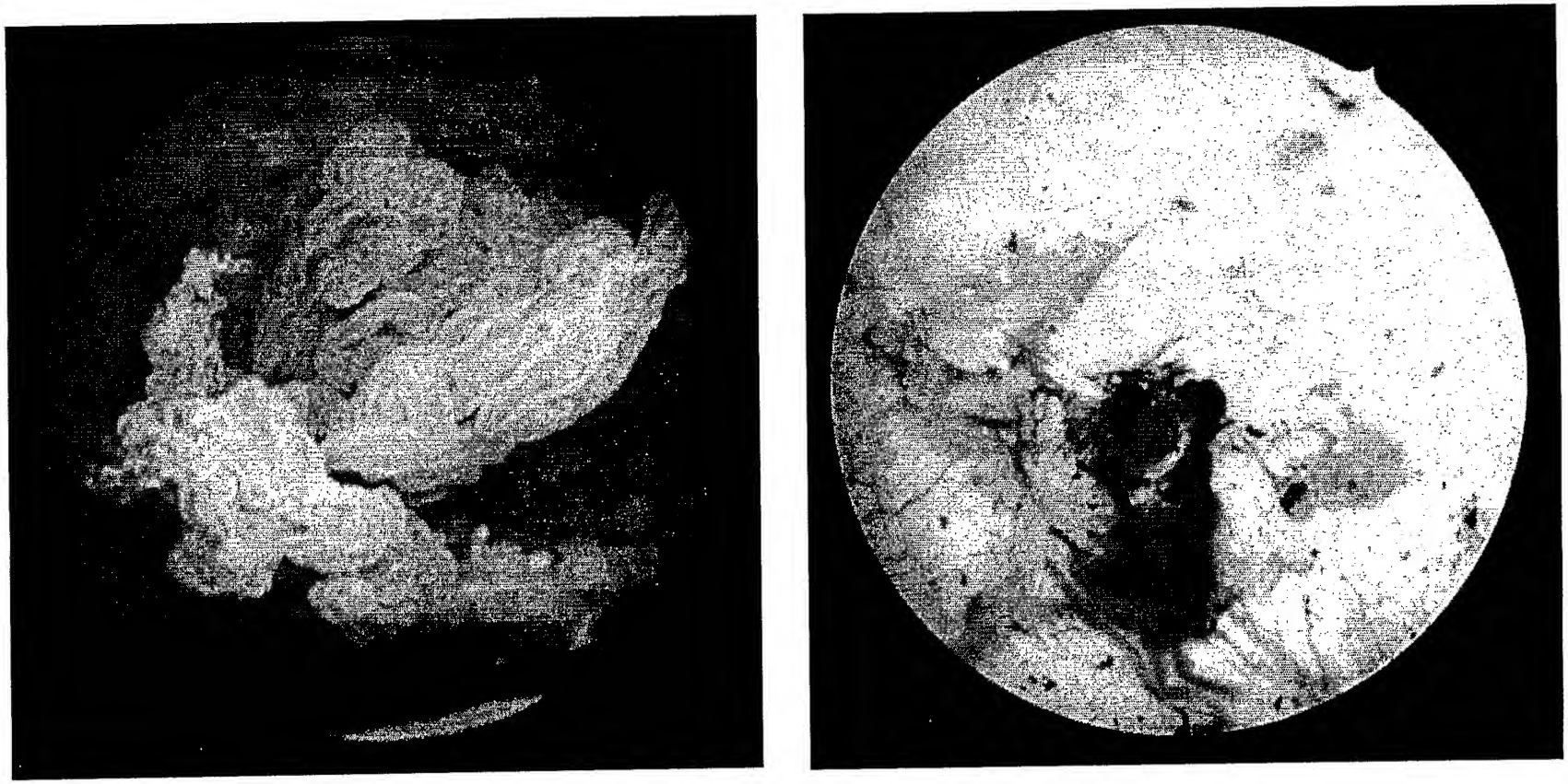
20. The use of a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the treatment of malignant skin tumours, in particular melanoma.

21. A method for treating malignant melanoma, which method comprises applying a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, to the melanoma.

Abstract

Therapeutic Treatment

The use of a biologically active complex of α-lactalbumin, selected from HAMLET (human α-lactalbumin made lethal to tumour cells) or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the treatment of mucosal cancers such as bladder cancer or malignant melanoma.



A B

P.S. *Before / After*

Figure 1